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(54) Title: METHODS FOR THE DETECTION OF ANTIGEN-SPECIFIC IMMUNE COMPLEXES

(57) Abstract

A simple and sensitive method is described for the detection of circulating immune complexes (IC's) in an antigen-specific manner. The method is based on the use of anti-peptide antibodies as solid-phase capture reagents to bind antigen which is complexed to serum antibodies. The bound serum antibody is detected with a labelled second antibody. The method requires that the anti-peptide antibodies bind native protein efficiently, and that the anti-peptide antibodies do not compete with antibodies raised against the native protein which are involved in IC formation. Two anti-peptide antibodies specific for the hepatitis B surface antigen (HBsAg) and the hepatitis B e antigen (HBeAg), which possessed the requisite characteristics, were chosen as models for IC assay development. The solid-phase, anti-peptide based assays efficiently detected HBsAg and HBeAg-containing IC's in preformed antigen/antibody mixtures and in the serum of chronically infected hepatitis B patients.

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METHODS FOR THE DETECTION OF
ANTIGEN-SPECIFIC IMMUNE COMPLEXES

Technical Field

The present invention relates to immunological methods for the detection of circulating immune complexes through the use of antibodies as solid-phase capture reagents that bind antigen complexed to serum antibodies.

Background

Historically, assays developed to measure circulating immune complexes (IC's) have emphasized detection of the immunoglobulin (Ig) component and consequently are antigen nonspecific (i.e., Clq binding, Raji cell assay, C3 binding). Antigen-specific IC assays are generally too complex for routine clinical performance.

A unique characteristic of HVB infection is the secretion into the serum of excess subviral proteins, which attain serum concentrations far greater than the virion itself. See, Heermann et al., "Surface Proteins of Hepatitis B Viruses". In: A. McLachlan (Ed.), Molecular Biology of the Hepatitis B Virus, CRC Press, pp 109 (1991). These secreted viral proteins may function in evading the anti-viral immune response during chronic infection. See, Milich et al., Proc. Natl., Acad., Sci., USA, 87:6599 (1990) and Milich et al., Proc. Natl., Acad., Sci., USA, 88:4348 (1991).

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Additionally, the presence of subviral proteins
including the hepatitis B surface antigen (HBsAg) and
the hepatitis B e antigen (HBeAg) in the serum affect
the ability to detect circulating antibodies and may
5   obscure the onset of seroconversion. The available
commercial assays usually detect anti-HBs and anti-HBe
antibodies only after the respective antigens have
been cleared from the serum. In HBeAg-expressing
transgenic mice, the observation was recently made
10   that anti-HBe antibodies could be detected even in the
presence of HBeAg using a direct binding antibody
assay [Milich et al., Proc. Natl., Acad., Sci., USA,
87:6599 (1990)]. However, detection of anti-HBe in
the presence of a vast excess of serum HBeAg or
15   detection of HBeAg in the presence of an antibody
excess was more problematic.

A number of investigators have reported the
presence of circulating immune complexes in patients
with HBV infection. Both antigen non-specific (U.S.
20   Patent Nos. 4,757,024 and 4,753,893, Abrass et al.,
Clin. Exp. Immunol., 40:292 (1980); Anh-Tran et al.,
J. Immunol. Methods, 33:293 (1980); Anh-Tran et al.,
Clin. Exp. Immunol., 43:246 (1981); and Thomas et al.,
Clin. Exp. Immunol., 31:150 (1978) and antigen-
25   specific (Sallberg et al.,Clin. Exp. Immunol., 84:116
(1991); Rath et al., Clin. Exp. Immunol., 72:164
(1988); Sagnelli et al., Liver, 5:205 (1985); Brown et
al., Immunol., 49:673 (1983); Brown et al., Clin. Exp.
Immunol., 55:355 (1984); and Pernice et al., J.
30   Immunol. Methods, 28:33 (1979) methods of IC detection
have been utilized. The obvious limitation of the
non-specific IC assays is the lack of information
regarding the viral and/or subviral origin of the
antigen component of the IC. The antigen-specific IC
35   assays are either difficult and cumbersome to perform

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(ie. PEG precipitation, IC disassociation followed by
soluble antigen assay) or sensitive over only a narrow
range of antigen:antibody ratios (ie. solid-phase
assays which utilize monoclonal or polyclonal
5   antibodies specific for the native antigens to capture
the complexed antigen). The limitation of the
previously described solid-phase capture methods is
that the antibodies used to capture the IC's are often
crossreactive with the serum antibodies involved in IC
10   formation and therefore compete for antigen binding.
See U.S. Patent Nos. 4,617,262 and 4,464,165. These
types of IC assays can detect IC's only in slight
antigen excess and function best in the context of
multivalent antigens (Rath et al., Clin. Exp.
15   Immunol., 72:164 (1988).

     For this reason, the method to detect circulating
immune complexes in an antigen-specific manner
described herein was developed to avoid these
complications. The method is antigen-specific,
20   sensitive in conditions of either antigen or antibody
excess, and is simple to perform. Furthermore, the
use of anti-peptide antibodies to design solid-phase
IC assays is not limited to the HBV system because the
method is applicable to any pathogen or auto-antigen
25   to which non-competing anti-peptide antibody can be
generated.

     Anti-peptide antibodies specific for two HBV
antigens, the HBeAg and HBsAg, were utilized. A
consistent feature (often thought of as a failing) of
30   antibodies raised against peptide fragments has been
that a high percentage of anti-peptide antibodies
which recognize the native protein often bind at
unique sites not relevant to the sites recognized by
antibodies raised to the intact protein. See, Milich,
35   "Synthetic T and B Cell Recognition Sites:

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Implication for Vaccine Development". Advances In
Immunology. Academic Press, pp 195 (1989). This
characteristic of anti-peptide antibodies is
advantageous since it makes them ideal candidates to
5   function as capture reagents for antigens already
complexed to serum antibodies directed to the native
protein. Therefore, anti-peptide antibodies that bind
native HBeAg or HBsAg were selected, but that do not
compete with antibodies raised against the native
10   antigens, to capture these serum antigens on a
solid-phase whether the antigen is free or
immune-complexed. The presence of Ig bound to the
captured antigen is determined by probing with a
peroxidase-labelled monoclonal anti-human IgG reagent.
15

Brief Summary of the Invention

        The present invention provides a method of
assaying circulating IC's based on the use of
solid-phase anti-peptide antibodies by a simple;
20   antigen-specific method.

        Therefore, the present invention describes a
method for detecting the presence of an immunocomplex
of antibody molecules immunocomplexed with a
preselected antigen in a sample. The immunocomplex
25   typically represents circulating immunocomplexes in a
patient experiencing an immune response, the detection
of which immunocomplex provides a clinician with
important diagnostic information regarding the health
of the patient.

30   The method comprises the steps of:

        a)   admixing the sample with a detecting
antibody molecule to form an immunoreaction admixture,
where the detecting antibody molecule is capable of
immunoreaction with the immunocomplex at a preselected
35   site of the immunocomplex;

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        b)   maintaining the immunoreaction admixture under immunoreaction conditions sufficient for the detecting antibody molecule to immunoreact with the immunocomplex and form a detecting antibody-immunocomplex reaction product; and

        c)   detecting the presence of the detecting antibody-immunocomplex reaction product, and thereby the immunocomplex.

        In preferred embodiments, the method is practiced in a solid phase format, with the detecting antibody attached to a solid support, thereby allowing the rapid detection of immunocomplexes.

        In preferred embodiments, the sample is a human body fluid sample, typically blood or serum.

        In preferred methods, the invention detects immunocomplexes having a preselected antigen selected from the group of antigens consisting of bacterial, viral, parasitic, fungal, tumor and self antigens. Preferred are the viral antigens selected from the group of viruses consisting of hepatitis B virus (HBV), human immunodeficiency virus, influenza A virus, Epstein-Barr virus, togavirus and rubella virus. Particularly preferred are the HBV antigens HBsAg and HBeAg.

        In one embodiment, the method uses a detecting antibody which is directed to a preselected epitope on the antigen defined by a polypeptide. In this embodiment, the detecting antibody is an anti-polypeptide antibody. Preferred are anti-polypeptide antibodies that immunoreact with the hepatitis B virus (HBV) antigen HBsAg and immunoreact with a polypeptide having an amino acid residue sequence shown in SEQ ID NO 1, or that immunoreact with the hepatitis B virus (HBV) antigen HBeAg and immunoreact with a polypeptide having an amino acid residue sequence shown in SEQ ID

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NO 2.

Brief Summary of the Drawings

        In the drawings forming a portion of this
disclosure:

        Figure 1 illustrates the solid-phase anti-peptide
specifically binds soluble native antigens. The IgG
fractions of either anti-HBs 125-137 (Figure 1A) or
anti-HBe 73-87 (Figure 1B) antisera were coated on the
solid-phase and dilutions of soluble HBsAg (open
circles) or HBeAg (closed circles) were added. Bound
soluble antigen was detected by peroxidase-labelled
human anti-HBs or human anti-HBe, and is expressed as
absorbance (OD_{492}) corrected for absorbance in the
absence of antigen.

        Figure 2 illustrates the kinetics of antibody
seroconversion and IC formation in a case (N.Y.) of
resolving chronic hepatitis B infection (Figure 2A,
upper panel). Serum samples were collected from 3/81
through 6/83 and analyzed for HBV DNA (dot
hybridization); HBeAg, HBsAg, a-HBe and a-HBs
(commercial assays, Abbott); and ALT levels (unit/l).
The same samples were assayed for anti-HBs (open
circles) by direct EIA and HBsAg/ICs (closed circles)
in the antigen-specific IC assay using anti-HBs
125-137 as the capture antibody shown in Figure 2B,
middle panel; and anti-HBe (open squares) by direct
EIA and HBeAg/ICs (closed squares) in the
antigen-specific IC assay using anti-HBe 73-87 as the
capture antibody. The data are expressed as a P/N
ratio, which represents the absorbance (OD_{492}) value of
the sample (P) as compared to the mean absorbance
value of 20 negative control sera (N).

        Figure 3 illustrates the kinetics of antibody
seroconversion and IC formation in a case (E.I.) of

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resolving chronic hepatitis B infection (Figure 3A,
upper panel). Serum samples were collected from 1975
through 1983 and analyzed for HBV DNA (dot
hybridization); HBeAg, HBsAg, a-HBe and a-HBs

5   (commercial assays, Abbott); and ALT levels (unit/l).
The same samples were assayed for anti-HBe (open
squares) by direct EIA and HBeAg/ICs (closed squares)
in the antigen specific IC assay using anti-HBe 73-87
as the capture antibody. The data are expressed as

10   P/N ratio, which represents the absorbance (OD_{492})
value of the sample (P) as compared to the mean
absorbance value of 20 negative control sera (N).

Detailed Description of the Invention

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A.   Definitions
          "Amino acid residue" refers to an amino acid
formed upon chemical digestion (hydrolysis) of a
polypeptide at its peptide linkages. The amino acid

20   residues described herein are preferably in the "L"
isomeric form. However, residues in the "D" isomeric
form can be substituted for any L-amino acid residue,
as long as the desired functional property is retained
by the polypeptide. NH_2 refers to the free amino

25   group present at the amino terminus of a polypeptide.
COOH refers to the free carboxy group present at the
carboxy terminus of a polypeptide. In keeping with
standard polypeptide nomenclature (described in J.
Biol. Chem., 243:3552-59 (1969) and adopted at 37 CFR

30   1.822(b)(2)), abbreviations for amino acid residues
are shown in the following Table of Correspondence:

                    TABLE OF CORRESPONDENCE
               SYMBOL   AMINO ACID
35   1-Letter   3-Letter

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Y	Tyr	tyrosine
G	Gly	glycine
F	Phe	phenylalanine
M	Met	methionine
A	Ala	alanine
S	Ser	serine
I	Ile	isoleucine
L	Leu	leucine
T	Thr	threonine
V	Val	valine
P	Pro	proline
K	Lys	lysine
H	His	histidine
Q	Gln	glutamine
E	Glu	glutamic acid
Z	Glx	Glu and/or Gln
W	Trp	tryptophan
R	Arg	arginine
D	Asp	aspartic acid
N	Asn	asparagine
B	Asx	Asn and/or Asp
C	Cys	cysteine
J	Xaa	Unknown or other

The term "antibody" in its various grammatical
forms is used herein as a collective noun that refers
to a population of immunoglobulin molecules and/or
immunologically active portions of immunoglobulin
molecules, i.e., molecules that contain an antibody
combining site or paratope.

An "antibody combining site" is that structural
portion of an antibody molecule comprised of heavy and
light chain variable and hypervariable regions that
specifically binds antigen.

The phrase "antibody molecule" in its various

grammatical forms as used herein contemplates both an
intact immunoglobulin molecule and an immunologically
active portion of an immunoglobulin molecule.

Exemplary antibody molecules are intact
immunoglobulin molecules, substantially intact
immunoglobulin molecules and those portions of an
immunoglobulin molecule that contain the paratope,
including those portions known in the art as Fab,
Fab', F9ab')$_2$ and F(v).

Fab and F(ab')$_2$ portions of antibodies are
prepared by the proteolytic reaction of papain and
pepsin, respectively, on substantially intact
antibodies by methods that are well known. See for
example, U.S. Patent No. 4,342,566 to Theofilopolous
and Dixon. Fab' antibody portions are also well known
and are produced from F(ab')$_2$ portions followed by
reduction of the disulfide bonds linking the two heavy
chain portions as with mercaptoethanol, and followed
by alkylation of the resulting protein mercaptan with
a reagent such as iodoacetamide. An antibody
containing intact antibody molecules are preferred,
and are utilized as illustrative herein.

The phrase "monoclonal antibody" in its various
grammatical forms refers to a population of one
species of antibody molecule of determined (known)
antigen-specificity. A monoclonal antibody contains
only one species of antibody combining site capable of
immunoreacting with a particular antigen and thus
typically displays a single binding affinity for that
antigen. A monoclonal antibody may therefore contain
a bispecific antibody molecule having two antibody
combining sites, each immunospecific for a different
antigen.

As used herein, the term "biological assay
conditions" is sued for those conditions wherein a

molecule useful in this invention such as an antibody
binds to another useful molecule such as an antigen
epitope within a pH value range of about 5 to about 9,
at ionic strengths such as that of distilled water to
5   that of about one molar sodium chloride, and at
temperatures of about 4 degrees C to about 45 degrees
C.

The word "complex" as used herein refers to the
product of a specific binding agent-ligand reaction.
10   An exemplary complex is an immunoreaction product
formed by an antibody-antigen reaction.

A "specific binding agent" is a molecular entity
capable of selectively binding another molecular
entity or ligand. Exemplary specific binding agents
15   are paratopic molecules, complement proteins or
fragments thereof, S. aureus protein A. and the like.
Preferably, the specific binding agent binds its
ligand when the ligand is present as part of a
complex.

20   The word "antigen" is a polypeptide or protein
that is able to specifically bind to (immunoreact
with) an antibody and form an immunoreaction product
(immunocomplex). The site on the antigen with which
the antibody binds is referred to as an antigenic
25   determinant or epitope.

B.   Assay Methods
The present invention broadly contemplates a
method of detecting the presence of immune complexes,
30   preferably those antigen-specific immune complexes
circulating in serum.

In accordance with this method, to detect the
presence of any formed circulating immunocomplexes, a
portion of the aqueous protein compositions, typically
35   serum from a patient such as described in Example 5,

is admixed with a solid support capable of
specifically binding the immunocomplex, thereby
forming a solid/liquid phase (capturing reaction)
admixture.

5   Solid supports capable of specifically binding an
immunocomplex are typically comprised of a specific
binding agent affixed (operatively linked) to a solid
matrix. Preferably, the solid-phase affixed specific
binding agent is an antibody molecule capable of
10   immunoreacting with the immunocomplex. It should be
noted that an antibody capable of immunoreacting with
either the antigen or the antibody of the surface
immunocomplex can be used. Preferred antibody
compositions for use in the method of this invention
15   bind to the antigen at unique sites not relevant to
the sites recognized by antibodies raised to the
intact protein. The preferred antibodies, either
polyclonal or monoclonal antibodies, bind and capture
antigens already complexed to serum or native
20   antibodies directed to the native antigen. Preferred
antibodies for use in detecting circulating the
hepatitis B virus surface antigen (HbsAg) and the
hepatitis B e antigen (HbeAg) are anti-peptide
antibodies raised against the amino acid residue
25   sequence from 125 to 137 of HbsAg of HbsAg of subtype
adw and the amino acid residue sequence of HbeAg of
subtype agw from 73 to 87, respectively. The peptides
and their corresponding antibodies are described in
Examples 1 and 2.

30   Useful solid matrices are well known in the art.
Such materials are water insoluble and include the
cross-linked dextran available under the trademark
SEPHADEX from Pharmacia, Piscataway, NJ; agarose;
polyvinylchloride, polystyrene, cross-linked
35   polyacrylamide, nitrocellulose or nylon-based webs

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such as sheets, strips or paddles; or tubes, plates or
the wells of a microtiter plate such as those made
from polystyrene, polycarbonate or polyvinylchloride.

When present as part of a solid support, a
specific binding agent is typically affixed to a solid
matrix by adsorption from an aqueous medium, although
other modes of affixation, such as covalent coupling,
well known to those skilled in the art, can be used.

The solid/liquid phase admixture is maintained
for a time period sufficient for any immunocomplex
present in serum or another body fluid to be bound by
the solid support and thereby form a solid-phase bound
complex. Preferably, the maintained solid support is
then separated from any non-bound protein, typically
by washing.

Assaying the maintained solid support for the
presence of solid-phase bound complex thereby provides
a means for detecting the presence of the
immunocomplex of interest in the aqueous sample. Such
assaying is typically performed by forming a labeling
reaction admixture by admixing the maintained solid
support with a labeled specific binding agent capable
of specifically binding the immunocomplex portion of
the solid-phase complex. Preferably, the labeled
specific binding is capable of binding either the
antigen or the antibody of the surface immunocomplex
when the surface immunocomplex is itself bound to the
solid support. More preferably, the solid-phase bound
complex is detected with an anti-IgG antibody
containing a labeling reagent, such as horseradish
peroxidase.

The labeling reaction admixture is maintained for
a time period sufficient for the labeled specific
binding agent to react with (specifically bind) any
solid-phase immunocomplex present and thereby form a

labeled solid-phase product. Any non-bound labeled
specific binding agent is then typically separated
from the solid support.

The presence of any labeled solid-phase product
5   formed is then determined by known assay procedures
that depend, as is well known, on the type of label
used. The presence of labeled solid-phase product
indicates the presence of the immunocomplex of
interest in the aqueous sample.

10   As used herein, the term "circulating
immunocomplex" means a complex formed of an antibody
bound to a circulating antigen epitope whose presence
is sought. The immunocomplex can be present in the
aqueous sample analyzed as obtained from a patient

15   (donor) as where the sample is a serum sample from a
patient such as a patient with hepatitis B virus
infection that contains autoantibodies to a hepatitis
B viral secreted proteins, such as HbsAg and HbeAg.
The immunocomplex to be assayed by the methods of this

20   invention includes any immunocomplexes circulating in
blood for which detection of specific antigens are
desired for serological analysis, such as with other
acute or chronic viral bacterial, or fungal, or
parasitic infections. Thus, for detection of various

25   immunocomplexes found in serum and the like,
antibodies which recognize the immunocomplex,
preferable the antigen, that do not bind at the same
epitope as the serum antibodies, are required as
described in the following section. Also contemplated

30   for use in this invention is the detection of an
antibody-antigen complex preformed in vitro by
reacting the antibody with the antigen epitope to form
soluble immunocomplexes such as though described in
Examples 7 and 8.

35   In preferred practice, sera from patients is

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collected for subsequent assaying with the methods of
this invention. The serum containing immunocomplexes
is thereafter affixed to a solid phase support to form
a solid phase-affixed immunocomplex. The affixation

5   can be physical adsorption, immunoreaction using solid
phase-bound antibodies directed to the antibodies of
the immunocomplex, such as those anti-peptide
antibodies described in Example 2.
        The reaction immunoreaction admixture is

10   maintained for a time period sufficient for an
immunocomplex containing the antibody and the cell
surface antigen to form on the cellular surface. As
is well known, the time period required for
immunocomplex formation depends on a variety of

15   factors including temperature and concentration of
reactants. Typically, the time period is
predetermined for a given set of reaction conditions
by well known methods prior to performing the assay.
Under biological assay conditions, the maintenance

20   time period is usually from minutes to hours, such as
30 minutes to about 2 hours. The immunocomplex is
thereafter assayed for as a solid phase-affixed
immunocomplex.
        The assay methods of the present invention

25   utilize a solid support capable of specifically
binding a solubilized immunocomplex. Useful solid
supports are typically comprised of a specific binding
agent affixed (operatively linked) to a solid matrix.
        A specific binding agent can be linked to a label

30   or indicating means and used to form a labeled
immunoreaction product.
        The terms "indicating means" and "label" are used
herein to include single atoms and molecules that are
capable of producing a detectable signal and of being

35   linked to an antibody or used separately.

15

The indicating means can be a fluorescent
labeling agent that chemically binds to antibodies or
antigens without denaturing them to form a
fluorochrome (dye) that is a useful immunofluorescent
5   tracer. Suitable fluorescent labeling agents are
fluorochromes such as fluorescein isocyanate (FIC),
fluorescein isothiocyanate (FITC), 5-dimethylamin-1-
naphthalenesulfonyl chloride (DANSC),
tetramethylrhodamine isothiocyanate (TRITC),
10   lissamine, rhodamine 8200 sulphonyl chloride (RB 200
SC) and the like.

In preferred embodiments, the indicating group is
an enzyme, such as horseradish peroxidase (HRP),
glucose oxidase, or the like. Where the principal
15   indicating group is an enzyme such as HRP or glucose
oxidase, additional reagents are required to visualize
the fact that a complex (immunoreaction product) has
formed. Such additional reagents for HRP include
hydrogen peroxide and an oxidation dye precursor such
20   as diaminobenzidine. An additional reagent useful
with glucose oxidase is 2,2'-azino-di-(3-ethyl-
benzthiazoline-G-sulfonic acid) (ABTS).

Radioactive elements are also useful labeling
agents and are used illustratively herein.
25   An exemplary radiolabeling agent is a radioactive
element that produces gamma ray emissions. Elements
which themselves emit gamma rays, such as ^{124}I, ^{125}I,
^{128}I, ^{131}I, ^{132}I, and ^{51}Cr represent one class of gamma
ray emission-producing radioactive element indicating
30   groups. Particularly preferred is ^{125}I. Another group
of useful indicating groups are those elements such as
^{11}C, ^{18}F, ^{15}O and ^{13}N which themselves emit positrons.
the positrons so emitted produce gamma rays upon
encounters with electrons present in the animal's
35   body. Also useful is a beta emitter, such as

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^{111}indium.

    The labeling of proteinaceous specific binding
agents is well known in the art. For instance,
antibodies produced by hybridomas can be labeled by
5   metabolic incorporation of isotope containing amino
acids provided as a component in the tissue culture
medium. See for example Galfre et al., <u>Meth.</u>
<u>Enzymol.</u>, 73:3-46 (1981). The techniques of protein
conjugation or coupling through activated functional
10   groups are particularly applicable. See, for example,
Aurameas, et al., <u>Scand. J. Immunol.</u>, Vol. 8, Suppl.
7:7-23 (1978) and U.S. Patent No. 4,493,795 which is
incorporated herein by reference. Method for
conjugating enzymes to proteins may be found in U.S.
15   Patent No. 3,791,932 and No. 3,839,153. In addition,
site directed coupling reaction can be carried out so
that the label does not substantially interfere with
the immunoreaction of the second receptor with apo B-
100. See, for example, Rodwell et al., <u>Biotech.</u>,
20   3:889-894 (1985).

      C.   <u>Immune Complexes</u>

    An immune complex (also referred to as IC)
as defined for detection by use of the methods of the
25   present invention consists of an antigen complexed
with an antibody that immunoreacts with an
immunodominant epitope on the antigen. Preferably the
immune complex is circulating in the blood but also
contemplated is an immune complex located
30   extravascular tissues. Further contemplated for
detection is an immune complex present on the surface
of a cell, such as that seen in patients with subacute
sclerosing panencephalitis where the patients'
antibodies to the measles virus envelope glycoproteins
35   are complexed with viral antigens on the surface of

the virally infected cell. These types of immune
complexes are defined as native. Also contemplated
for use with the methods described herein are immune
complexes that are nonnative such as those preformed

5   _in vitro_ by the admixture of antigen and antibody such
as those described in Examples 7 and 8.

The formation of immune complexes circulating in
the blood depend on the relative proportions of
antigen and antibody and also on antibody affinity.

10 With an excess of antibody, each antigen molecule is
bound with antibody and is rapidly removed by
reticulendothelial cells. When equal amounts of
antigen and antibody are present, lattice structures
resulting in large aggregates are formed and

15 eventually removed. However, when antigen
concentration is in excess or serum-derived antibodies
are of lower affinity, the formed complexes are not
removed from the circulation.

Those immune complexes that remain in the

20 circulation are formed in most acute viral infections
where after virus enters the blood stream, an immune
response is generated. This state persists until
antibody responses increase or greater affinity
antibodies combine with the antigen. Under those

25 circumstances, the resultant complexes are deposited
in blood vessels and in liver glomeruli over the
course of long term viral infection causing release of
viral antigens into the blood as is a unique
characteristic of hepatitis B virus (HBV) infection.

30 Subviral proteins are secreted into the serum and
attain serum concentrations in excess of intact viral
concentrations. Circulating immune complexes are also
deposited in the walls of small blood vessels in the
skin and joints where subsequent inflammatory

35 reactions occur. Viral infections where immune

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complexes are formed besides HBV and measles include Epstein-Barr virus, togavirus, human immune deficiency virus and influenza A virus.

The antibodies generated against viral, bacterial, fungal, self, parasitic or tumor-derived antigens and the like recognize the immunodominant epitope on that antigen. What is meant by immunodominant epitope is that antibodies are generally raised against and recognize the native conformation of a protein or antigen. For instance, antibodies to native staphyloccal nuclease were found to have an increased affinity of approximately 5000-fold to the native protein that for the corresponding polypeptide on which they were isolated. Thus, the specificity with the generation of the immune response of this type is dependent not only on the antigenic determinant based on a specific sequence of amino acid residue sequences but more importantly on the three dimensional conformation of those residues constrained in space. The antibodies complexed with circulating antigens such as those recognizing hepatitis B viral surface and e antigens have been shown to bind to specific sites on the antigen.

For detection of the immune complexes as performed by the methods of the present invention, the antibody reagent used for binding to the immune complex must then necessarily not be directed in specificity towards the same site as those recognized by those found in serum. More specifically, the antibody must recognize a nondominant site on the antigen comprising the immune complex. In addition, the antibody must be able to bind to the immune complex without any steric hindrance imposed by the immune complex itself. Thus, for the detection of

immune complexes, an antibody reagent having such a
specificity is required. This reagent is referred to
as the detecting reagent or detecting antibody. The
detecting antibody thus functions as a capture reagent

5   for the immune complexes, without the binding of which
no detection would occur. Therefore detecting
antibody is synonymous with capture antibody.

Contemplated for use as detecting antibodies are
antibody reagents including polyclonal and monoclonal

10   antibodies raised against intact antigen immunogens,
polyclonal or monoclonal antibodies raised against
preferred polypeptide sequences of the antigen; and
monoclonal antibodies that recognize intact antigen or
derived polypeptides prepared from recombinant

15   combinatorial library techniques such as those
prepared as described by Persson et al., *Proc. Natl.*
Acad. Sci., USA, 88:2432-2436 (1992) and Barbas et
al., *Proc. Natl. Acad. Sci., USA*, 88:7978-7982 (1992).

In addition to the detecting antibody having the

20   specificity for a nondominant site on the antigen in
the immune complex is a detecting antibody having the
specificity for a native antibody produced in response
to a foreign antigenic load. Thus, the detecting
antibody is typically an anti-idiotypic antibody.

25   Circulating immune complexes have been identified
in hepatitis B virus infections, other viral
infections previously mentioned, various lung diseases
(cryptogenic fibrobosing alveolitis, chronic
interstitital pneumonia, fulminating pulmonary edema,

30   and chronic bronchitis), in sarcodoisis, in several
collagen-vascular diseases such as rheumatoid
arthritis, in leprosy, in retinal vasculitis and in
schitosomiasis to name a few. In order to detect
immune complexes such as those listed herein, a

35   detecting reagent, preferably a detecting antibody,

20

must be produced, and if already produced, must be
screened for the ability to bind to the immune complex
at a site that is not inhibited by the antibody
comprising part of the immune complex. Detecting
5   antibodies can be raised as described herein against
the intact antigen in question or a specified
polypeptide sequence comprising a part of the antigen.
Amino acid residue sequences of typical antigens such
as those listed herein are available through databases
10   such as GenBank. Defined polypeptides can then be
used as immunogens as described in Example 1 for
hepatitis B virus surface and e antigens. The
resultant antibodies are then screened for
immunospecificity. Typical screening assays for
15   determining the binding specificity of detecting
antibodies include binding and inhibition of binding
assays such as those described in Examples 5 and 6.
With such an approach, any potential detecting reagent
can be obtained to allow for the detection of an
20   immune complex.

Examples

        The following examples relating to this invention
are illustrative and should not, of course, be
25   construed as specifically limiting the invention.
Moreover, such variations of the invention, now known
or later developed, which would be within the purview
of one skilled in the art are to be considered to fall
within the scope of the present invention hereinafter
30   claimed.

1.   Preparation of Recombinant Antigens and Synthetic
       Peptides
       Recombinant hepatitis B surface antigen (HBsAg)
35   of the adw subtype was provided by G. Bitter (Amgen,

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Thousand Oaks, CA). Recombinant hepatitis B e antigen
(HBeAg) of the ayw subtype was provided by S. Stahl
(Stahl et al., _Proc. Natl. Acad. Sci., USA_, 79:1606
(1982); Milich et al., _J. Immunol._, 141:3617 (1988)).

5   Synthetic peptides were synthesized by the Merrifield
solid-phase method, and were subjected to HPLC on a
C18 reverse phase column. All peptides eluted as a
single major peak (>90%). Synthetic peptides were
produced in the peptide laboratory of the R.W. Johnson

10   Pharmaceutical Research Institute, La Jolla, CA, and
were provided by K. Hoey. The HBsAg-derived peptide
utilized is comprised of amino acids 125-137 of
HBsAg/adw and is designated as HBs 125-137:
TTPAQGNSMFPSC (SEQ ID NO 1) [Alexander et al.,

15   "Chemically Synthesized Peptide Analogues of the
Hepatitis B Surface Antigen. In: F.V. Chisari (Ed.),
Advances in Hepatitis Research, pp 223 (1984)]. The
HBeAg-derived peptide utilized is comprised of amino
acids 73-87 (exclusive of the precore sequence) of

20   HBeAg/ayw and is designated as HBe 73-87:
GVNLEDPASRDLVVSC (SEQ ID NO 2).

2.   _Preparation of Antibodies_

     To produce anti-peptide antibodies, peptides HBs
25   125-137 and HBe 73-87 were coupled to keyhole limpet
hemocyanin (KLH) using glutaraldehyde. Rabbits were
immunized subcutaneously with 200 μg of the
peptide-KLH conjugate emulsified in complete Freund's
adjuvant (CFA), and were boosted with an equal dose of

30   peptide conjugate in incomplete adjuvant. Antisera
were collected and IgG fractions were obtained by ion
exchange chromatography (Bio-Rad Econo-Pac 10 DG).
The concentration of anti-HBs 125-137 was 1.0
milligrams/milliliter (mg/ml) and anti-HBe 73-87 was

35   4.3 mg/ml.

22

Human polyclonal anti-HBs and anti-HBe antisera
were obtained from recovered HBV infected patients.
Murine polyclonal anti-HBs and anti-HBe were produced
by immunization with recombinant antigens. Polyclonal
5   rabbit anti-HBe was purchased (DAKO Corporation, CA),
and the monoclonal anti-human IgG was provided by
Ortho Diagnostics, NJ.

3.   Solid-Phase Direct Enzyme Immunoassays
10   Direct solid-phase enzyme immunoassays (EIA) were
used to measure free anti-HBs and free anti-HBe in
serum and in antigen/antibody mixtures in vitro.
Recombinant HBsAg or HBeAg diluted in 0.01 M
bicarbonate buffer, pH 9.6 were coated onto microtiter
15   plates (50 nanograms (ng) in each well) overnight at 4
degrees Celsius (4°C). The plates were washed 5x in
phosphate-buffered saline (PBS) with 0.1% bovine serum
albumin (BSA) and .005% Tween 20 (wash buffer). The
plates were preincubated for 1 hour at 37°C with 50
20   microliter (ul) of PBS containing 1% BSA, 0.005% Tween
20 and 5% heat inactivated goat serum (blocking
buffer) to reduce nonspecific binding. The sera to be
tested for anti-HBs or anti-HBe reactivity were
diluted 1:50 in blocking buffer and added to the
25   solid-phase antigen plates and incubated for 2 hours
at 37°C. After washing, 50 ul of peroxidase
conjugated monoclonal anti-human IgG (1:2000) was
added and the plates were incubated for 2 hours at
37°C. The plates were developed by a final incubation
30   for 10 minutes with 50 ul of orthophenylene diamine
(OPD). The absorbance was read on an automatic
microtiter plate reader (Dynatech; MR600) and results
are expressed as optical density (O.D.) units at 492
nm. All EIA's were performed on at least three
35   occasions, and the experiments shown are

representative.

4.   Quantitative Antigen-Specific Immune Complex
     Assays

5   To detect antigen-specific immune complexes in
serum or in antigen/antibody mixtures in vitro,
solid-phase EIA's were developed. To detect
HBsAg-containing IC's, microtiter plates were coated
with an IgG fraction of anti-HBs 125-137 (0.5
10   microgram (ug) in each well), and to detect
HBeAg-containing IC's, plates were coated with an IgG
fraction of anti-HBe 73-87 (1.0 ug/well) in 0.01 M
bicarbonate buffer, pH 9.6 overnight at 4°C. The
plates were then washed and blocked and the sera to be
15   tested were diluted 1/50 in blocking buffer and added
to the anti-peptide coated plates. The plates were
incubated for 2 hours at 37°C, washed and developed
with the peroxidase-labelled Mab anti-human IgG as
described.

20

5.   Patients
         Twenty patients who were consistently positive
for HBsAg and HBeAg in serum for more than 1 year and
were diagnosed as chronic active hepatitis (CAH) were
25   studied. All sera had been tested for HBsAg/anti-HBs,
HBeAg/anti-HBe, anti-HBc, IgM-anti-HBc, and anti-HD
using commercial enzyme immunoassays (EIA's; Abbott
Laboratories, IL). Hepatitis B virus DNA (HBV-DNA)
was determined using a dot blot procedure. Human
30   anti-HBs positive sera were obtained from patients who
had recovered from acute hepatitis B, and human
anti-HBe positive sera were obtained from patients who
had been chronically infected and subsequently
seroconverted to anti-HBe positive. In each assay at
35   least 15 human sera, which were negative for all HBV

markers, were used as negative controls. The mean
plus 2 standard deviations of the absorbance values of
the control sera was used as the cut off value in all
assays. Statistical analyses were performed using a
5   standard chi-square (X2) test.

6.   Characterization of Anti-Peptide Antibodies
        In order for an anti-peptide antibody to be
useful as a reagent capable of capturing circulating
10   IC's several criteria were first satisfied. First,
the anti-peptide reagent must efficiently bind the
native protein antigen in a specific manner. Anti-HBs
125-137 has been shown previously to bind native
HBsAg/adw [Alexander et al., "Chemically Synthesized
15   Peptide Analogues of the Hepatitis B Surface Antigen.
In: F.V. Chisari (Ed.), Advances in Hepatitis
Research, pp 223 (1984)] and as illustrated in Table I
efficiently binds HBsAg but not HBeAg or the
particulate nucleocapsid antigen (HBcAg). The
20   HBeAg-derived peptide 73-87 was selected from a panel
of non-overlapping peptides because immunization with
this sequence elicited the highest titer antibody
response to native HBeAg and HBcAg (Table I).
Anti-HBe 73-87 bound HBcAg as well as HBeAg because
25   this amino acid sequence is shared between HBeAg and
HBcAg, and is obviously accessible on the surface of
both proteins. However, circulating immune complexes
are more likely to contain HBeAg as opposed to HBcAg
because HBeAg is a secreted protein whereas HBcAg
30   accumulates intracellularly in infected hepatocytes
and is only secreted as an internal component of the
intact virion.

Table I.   Specificity of Anti-peptide Antisera
for Native Antigens

Anti-peptide	Antibody Titer		
	HBsAg	HBeAg	HBcAg
Anti-HBs 125-137	1:25,600	0	0
Anti-HBe 73-87	0	1:102,400	1:409,600

Solid-phase recombinant antigens were coated on
plastic wells (50 ng/well) and were incubated with
4-fold dilutions of the rabbit anti-peptide antisera.
Titers are expressed as the highest dilution to yield
3x the OD_{492} reading of preimmunization sera.

Because the design of the IC assay is based on a
solid-phase capture format, immobilized anti-peptide
antibody must be capable of binding native antigen in
solution. For this purpose, IgG fractions of rabbit
anti-HBs 125-137 and anti-HBe 73-87 were prepared and
coated onto microtiter wells, and the ability to bind
either HBsAg or HBeAg in solution was determined. The
results are shown in Figure 1. Anti-HBs 125-137
quantitatively bound HBsAg but not HBeAg (Figure 1A),
and reciprocally anti-HBe 73-87 bound HBeAg but not
HBsAg (Figure 1B). The bound native antigens were
detected with polyclonal human antibodies labelled
with peroxidase. The fact that native antigen
epitopes were still available for binding by the
labelled antibodies suggested that the anti-peptide
antibodies bound the native antigens at unique sites.
The absence of competitive binding between
anti-native and anti-peptide antibodies is another
characteristic that is required of an anti-peptide
antibody that is to be used to capture immune-
complexed antigen. As shown in Table II, rabbit

anti-HBs 125-137 binding to HBsAg was not
significantly blocked by murine or human antibodies
raised against native HBsAg. In contrast, the control
rabbit anti-HBs antibody, raised against native HBsAg,

5   was blocked by both murine and human polyclonal
anti-HBs antisera. Similarly, anti-HBe 73-87 only
minimally competed with polyclonal murine and human
antibodies specific for the native HBeAg, whereas
control rabbit anti-HBe was significantly blocked by

10   the competitor antibodies (Table II). The minimal
competition between anti-HBe 73-87 and antibodies
raised against native HBeAg suggest a contribution of
residues 73-87 in a native HBeAg epitope as previously
suggested (Salfeld et al., J. Virol., 63:798 (1989).

15   However, the minimal inhibition indicates that the
linear peptide 73-87 does not fully represent an
entire native epitope, which most likely requires
greater conformational integrity. In support of the
conformational dependence of the epitope(s) involving

20   residues 73-87, none of the numerous HBcAg and
HBeAg-specific monoclonal antibodies we have examined
significantly bind to the 73-87 peptide.

27

Table II.   Anti-peptide Antibodies Do Not Compete
With Anti-HBs and Anti-HBe
for Binding to Native Antigen

Rabbit Antibody	Competitor Antibody (% Inhibition)							
	Murine a-HBs		Murine a-HBe		Human a-HBs		Human a-HBe	
	1:25	1:100	1:25	1:100	1:25	1:100	1:25	1:100
a-HBs	40	34	0	0	72	42	0	0
a-HBs 125-1379	0	0	0	14	0	0	0	0
a-HBe	0	0	86	74	0	0	42	27
a-HBe 73-87	0	0	28	10	0	0	7	2

Solid-phase HBsAg or HBeAg (50 ng/ml) were incubated
with dilutions of murine or human-derived competitor
antibodies or normal serum followed by the addition of
rabbit antibodies specific for the native antigens
(a-HBs/a-HBe) or the peptide antigens. The data is
expressed as percent inhibition by the competitor
antibodies relative to the inhibition in the presence
of normal murine or human serum.

Alternatively, the lack of competition between
the antibodies raised against the peptide antigens and
the antibodies raised against the native antigens
(Table II) may be due to differences in antibody
avidity. Because of the assumption that the
anti-peptide antisera may be of lower avidity for the
native antigens, the antibodies raised against the
native antigens were used as the competitors. The
failure of the higher avidity antibodies raised
against the native antigens to prevent the binding of
the anti-peptide antibodies to the native antigens
indicate that differences in antibody avidity do not
account for the lack of competition. Furthermore, the
antibody competition experiments were performed in

both directions and yielded similar results.
Therefore, the selected anti-peptide antibodies bind
the respective native antigens at unique sites which
do not significantly interfere with simultaneous
binding by antibodies raised against the native
proteins.

7.   Use of Solid-Phase Anti-HBe 73-87 To Detect
      Preformed HBeAg/Anti-HBe Immune Complexes

        The ability of solid-phase anti-HBe 73-87 to
efficiently bind native HBeAg in solution and to bind
at a unique site not competitive with antibodies
raised to the native HBeAg, suggested that anti-HBe
73-87 may represent an ideal reagent to bind HBeAg
complexed to human anti-HBe₁ To test this method,
HBeAg/anti-HBe immune complexes were prepared using
recombinant HBeAg and human polyclonal anti-HBe
antibodies at varying antigen and antibody
concentrations. Solid-phase anti-HBe 73-87 was used
as the capture antibody and bound HBeAg/anti-HBe IC's
were detected with a peroxidase-labelled monoclonal
anti-human IgG reagent. Analysis of a wide range of
HBeAg concentrations and at varying anti-HBe dilutions
(i.e., checkerboard analysis) allowed the sensitivity
of the IC assay to be determined in conditions of
antigen or antibody excess. The HBeAg concentrations
were chosen to approximate the range of serum
concentrations of HBeAg during HBV infection. The
results are shown in Table III.

Table III. Use of solid-phase anti-HBe 73-87 to
detect preformed HBeAg/anti-HBe immune complexes.

HBeAg (lg/ml)

	0	.0006	.003	.01	.04	.16	.64	2.5	10	40
Human a-HBe (dilution)										
0	0	.01	.01	.01	0	0	.01	.01	.01	0
1:2560	0	0	0	.02	.07	.06	.09	0	0	0
1:512	0	.03	.05	.08	.16	.29	.28	.15	.13	.03
1:128	0	.04	.13	.15	.31	.62	.67	.78	.27	.20
1:32	0	.04	.18	.27	.39	.78	1.1	.95	.46	.16
1:8	0	.07	.16	.22	.20	.61	.91	.87	.50	.16
1:32	0	0	0	0	0	.01	0	0	0	0
1:8	0	0	01	0	0	.02	.03	.02	.02	.01

The indicated concentrations of soluble HBeAg were
mixed with equal volumes of dilutions of a polyclonal
human anti-HBe or normal human serum (NHS), and the
preformed (ICs) were allowed to equilibrate overnight
at 4°C. The HBeAg/anti-HBe ICs were then added to
plastic wells coated with an IgG fraction of anti-HBe
73-87. The bound HBeAg/anti-HBe ICs were detected by
a peroxidase-labelled Mab specific for human Ig. The
data are expressed as absorbance (OD_{492}) corrected for
the absorbance value in the absence of HBeAg for each
antibody dilution. The boxed values are at least 3x
the NHS background.

As shown in Table III, the solid-phase assay
efficiently detected HBeAg/anti-HBe IC's over a wide
range of antigen and antibody concentrations. As
expected, the sensitivity of the solid-phase IC assay
varied as a function of HBeAg concentration at a given

anti-HBe dilution. For example, at an anti-HBe
dilution of 1:2560 only a concentration of 0.64 ug/ml
of HBeAg yielded detectable IC's, at an anti-HBe
dilution of 1:512 IC's were detected over a range of

5   HBeAg concentrations (0.04-10.0 ug/ml), and at
anti-HBe dilutions between 1:8 and 1:128 IC's were
detected at HBeAg concentrations from 0.003 to 40
ug/ml. Also note that optimum IC detection occurred
at intermediate HBeAg concentrations (.64-2.5 ug/ml)

10   and IC detection declined at either lesser or greater
HBeAg concentrations (Table III). Furthermore,
maximum IC detection occurred at an anti-HBe dilution
of 1:32 and not at the lower dilution of 1:8. These
results are consistent with the dependence of

15   HBeAg/anti-HBe IC formation on the antigen/antibody
ratio and the optimum ratio is intermediate between
antibody excess and antigen excess. The optimal HBeAg
concentration was 0.64 ug/ml in the context of an
anti-HBe dilution of 1:32.

20   It was also of interest to determine the ability
of a direct solid-phase anti-HBe assay to detect
"free" anti-HBe in the presence of soluble HBeAg. The
direct assay utilized HBeAg (50 ng/well) as the solid-
phase ligand and a peroxidase-labelled monoclonal

25   anti-human IgG as the second antibody. The preformed
HBeAg/anti-HBe IC's utilized in the solid-phase IC
assay were assayed for anti-HBe activity in the direct
EIA and by a commercial anti-HBe assay (Abbott) (Table
IV).

30

Table IV. Sensitivity of Direct Solid-Phase
Anti-HBe Assay in the Presence of Soluble HBeAg

HBeAg (lg/ml)									
0	.0006	.003	.01	.04	.16	.64	2.5	10	40

Human a-HBe (dilution)

dilution										
0	0	0	0	0	0	0	0	0	0	0
1:512	.15[a]	.07	.10	.08	.04	0	0	0	0	0
1:128	.77	.53	.59	.58	.46	.18	.03	.01	.01	0
1:32	1.2	1.1	1.1	1.0	.92	.65	.23	.04	.02	.02
1:8	1.7	1.6	1.4	1.4	1.3	1.1	.87	.28	.06	.05

Dilutions of a polyclonal human anti-HBe serum or
normal human serum (NHS) were mixed with an equal
volume of buffer or the indicated concentrations of
soluble HBeAg and allowed to equilibrate overnight at
4°C. The ability to detect free anti-HBe in the
mixtures was determined by ELISA. Solid-phase HBeAg
(50 ng/well) served as the ligand and a peroxidase-
labelled Mab anti-human Ig was used as the second
antibody.
[a]The superscripts refer to the absence (˙) or presence (*)
of anti-HBe as determined by the commercial
(Abbott) anti-HBe assay. The values to the left of
the line are at least 3x the NHS background.

     "Free" anti-HBe was detected by the direct EIA
assay as a function of the initial anti-HBe dilution
and the soluble HBeAg concentration, as expected. At
an anti-HBe dilution of 1:32, an HBeAg concentration
of 2.5 ug/ml (125 ng) was sufficient to neutralize the
detection of anti-HBe by the direct EIA, whereas, an
HBeAg concentration of 0.64 ug/ml (32 ng) was only
partially inhibitory. This is consistent with
approximately equivalent binding of anti-HBe to HBeAg

on the solid-phase and to HBeAg in solution inasmuch
as ¯50 ng of HBeAg was present on the solid-phase.
Note that the solid-phase IC assay appears more
sensitive in the context of high HBeAg concentrations
as compared to the direct EIA, suggesting that
anti-HBe is not able to dissociate from HBeAg
complexes and bind solid-phase HBeAg, at least at
higher antigen concentrations. Also note that the
direct anti-HBe EIA as well as the solid-phase IC
assay is significantly more sensitive than the
commercial anti-HBe assay. Most likely this results
from the fact that the commercial anti-HBe assay is a
competitive EIA.

8. Use of Solid-Phase Anti-HBs 125-137 to Detect
    Preformed HBsAg/Anti-HBs Immune Complexes
        Similar to the results obtained with anti-HBe
73-87, solid-phase anti-HBs 125-137 efficiently
captured HBsAg complexed to human polyclonal anti-HBs
over a range of antigen/antibody ratios (Table V). At
an anti-HBs dilution of 1:8 HBsAg/anti-HBs IC's were
detected at HBsAg concentrations from 0.01 ug/ml to 40
ug/ml. An HBsAg concentration of 100 ug/ml appeared
to represent a level of antigen excess which precluded
IC detection in this assay.

Table V. Use of Solid-Phase Anti-HBs 125-137 to
Detect Preformed HBsAg/anti-HBs Immune Complexes

HBsAg (μg/ml)								
0	.01	.04	.16	.64	2.5	10	40	100

Human a-HBs (dilution)

dilution	0	.01	.04	.16	.64	2.5	10	40	100
0	0	0	0	.01	0	0	0	0	0
1:512	0	.01	.01	.03	.04	.03	.01	0	0
1:128	0	.04	.04	.08	.19	.18	.16	.12	0

1:32	0	.11	.24	.34	.35	.37	.28	.14	0
1:8	0	.22	.24	.22	.21	.24	.83	.39	0
NHS									
1:32	0	0	0	0	0	0	0	0	0
1:8	0	0	.01	.02	0	.03	0	0	0

The indicated concentrations of soluble HBsAg were mixed with equal volumes of dilutions of a polyclonal human anti-HBs or normal human serum (NHS), and the preformed immune complexes (ICs) were allowed to equilibrate overnight at 4°C. The HBsAg/anti-HBs ICs were then added to plastic wells coated with an IgG fraction of anti-HBs 125-137. The bound HBsAg/anti-HBs ICs were detected by a peroxidase-labelled Mab specific for human Ig. The data are expressed as absorbance (OD_{492}) corrected for the absorbance value in the absence of HBsAg for each antibody dilution. The boxed values are at least 3x the NHS background.

However, the HBsAg/anti-HBs IC assay was clearly more sensitive than a direct binding anti-HBs assay, which was not able to detect "free" anti-HBs at HBsAg concentrations from 0.64 to 40 ug/ml depending on the initial anti-HBs dilution (Table VI).

Table VI. Sensitivity of Direct Solid-Phase Anti-HBs
Assay in the Presence of Soluble HBsAg

	HBsAG (μg/ml)								
	0	.01	.04	.16	.64	2.5	10	40	100
0	0	0	0	0	0	0	0	0	0
1:512	.35*ᵃ	.34*	.27*	.15*	.02	01	01	0	0
1:128	1.1*	1.0*	.90*	.65*	.32*	.03	.02	0	0
1:32	1.7*	1.6*	1.5*	1.5*	1.0*	.52*	.06	.02	0
1:8	1.9*	1.8*	1.7*	1.8*	1.7*	1.0*	0.8*	.12	0

34

Dilutions of a polyclonal human anti-HBs serum or
normal human serum (NHS) were mixed with an equal
volume of buffer or the indicated concentrations of
5   soluble HBsAg and allowed to equilibrate overnight at
4°C. The ability to detect free anti-HBs in the
mixtures was determined by ELISA. Solid-phase HBsAg
(50 ng/well) served as the ligand and a peroxidase-
labelled Mab anti-human Ig was used as the second
10   antibody.
*The superscripts refer to the absence (⁻) or presence
(⁺) of anti-HBs as determined by the commercial
(Abbott) anti-HBs assay. The values to the left of
the line are at least 3x the NHS background.
15

9.   Evaluation of Clinical Specimens Using the
     Solid-Phase IC Assays
     Although the anti-peptide based, solid-phase IC
assays performed well using premixed IC's, a useful IC
20   assay must detect "natural" IC's as they exist in
human sera. Therefore, the sera of 20 patients
diagnosed with HBsAg-positive, HBeAg-positive, chronic
active hepatitis (CAH) B were compared with the sera
of 20 healthy control subjects for the presence of
25   HBeAg-containing immune complexes (HBeAg/IC's) and
HBsAg-containing immune complexes (HBsAg/IC's) (Table
VII).

            Table VII. Detection of Anti-HBs and Anti-HBe
30   and Immune Complexes in Sera of Chronic Active Hepatitis B
            Patients Seronegative by Commercial Assay

Assay	Chronic Active Hepatitis (HBeAg+/HBsAg+)	Healthy Controls n = 20

35

n = 20

Anti-HBe (Direct EIA)	20/20[a] (0.45 % 0.2)	0/20 (.019 % .011)
Anti-HBe (Abbott)	0/20	0/20
HBeAg/ICs	8/20[a] (0.46 % 0.14)	0/20 (.17 % .07)
Anti-HBs (Direct EIA)	7/20[b] (0.41 % 0.34)	0/20 (.08 % .05)
Anti-HBs (Abbott)	0/20	0/20
HBsAg/ICs	9/20[a] (0.3 % 0.07)	0/20 (.11 % .05)

The sera of 20 patients with HBsAg +, HBeAg + chronic
active hepatitis B were compared with the sera of 20
healthy control subjects in the indicated assays. The
commercial (Abbott) anti-HBe and anti-HBs assays were
performed according to the manufacturers instructions
using undiluted sera. The direct EIA methods used to
detect anti-HBe and anti-HBs, and the methods to
detect antigen-specific immune complexes (ICs) are
described herein. The direct EIA's and IC assays
utilized a 1:50 dilution of sera. The number of
positive sera/number tested and the mean absorbance
(OD_{492}) plus s.d. are shown. An OD_{492} value greater
than the mean plus 2 s.d. of the absorbance of 20
control sera was designated as positive.
[a]CAH vs. controls, p < .01.
[b]CAH vs. controls, p < .05

Although all CAH patients were seronegative for
anti-HBe and anti-HBs by commercial assays (Abbott),
the direct anti-HBe EIA revealed that 20 of 20 CAH
sera actually possessed anti-HBe reactivity, and the
direct anti-HBs EIA detected 7 of 20 anti-HBs positive

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samples. This finding is consistent with another
recent report that anti-HBe can co-exist with HBeAg in
a proportion of CAH patients and can be detected with
a sensitive solid-phase anti-HBe assay (Sallberg et
al., 1991). Eight of 20 CAH patient sera also
exhibited HBeAg-containing IC's as measured by the
solid-phase IC method utilizing anti-HBe 73-87 as the
capture reagent. The failure to detect HBeAg/IC's in
a number of specimens containing both HBeAg and
anti-HBe may have been due to low HBeAg concentrations
($\leq$ 10 ug/ml) since sera were diluted 1:50 for the IC
assay due to occasional high backgrounds at lower
dilutions. We anticipate that the sensitivity of the
HBeAg/IC assay will be optimized by the use of a
monoclonal anti-peptide antibody instead of a rabbit
IgG fraction on the solid-phase. The HBsAg-specific
IC assay detected HBsAg/IC's in 9 of the 20 CAH
patient specimens, whereas, anti-HBs was measured in 7
of 20 by the direct EIA method. The greater
sensitivity of the HBsAg/IC assay as opposed to the
direct anti-HBs EIA may reflect the relatively high
serum concentrations of HBsAg ($\leq$ 100 ug/ml) in these
CAH patients, which adversely affects the direct EIA,
and may actually enhance the sensitivity of the IC
assay (Tables V and VI). Cumulatively, the combined
use of the direct EIA's and the antigen-specific,
solid-phase IC assays revealed the presence of active
humoral immune responses occurring in these CAH
patients, which are not apparent from analysis with
the conventional serological assays available
commercially.

10.   Evaluation of the Kinetics of Seroconversion and
      Immune Complex Formation
           Chronic HBV infection is not a static process,

and a major advantage to the development of
antigen-specific IC assays is the ability to monitor
the specificity and progression of the patients
underlying immune response. This information may aid
5   in the prognosis and/or recommendation for treatment
of CAH patients. The course of a CAH patient (N.Y.)
monitored for a 3 year period is shown in Figure 2.
The conventional serological analysis of patient N.Y.
is illustrated in the upper panel (Figure 2A). The
10   patient was HBV DNA-positive and HBeAg-positive
through 4/82 and HBsAg-positive throughout the
observation period; and seronegative for anti-HBe
until 3/83 and seronegative for anti-HBs throughout as
measured by commercial antibody assays. In contrast,
15   analysis for circulating IC's and direct antibody
analysis revealed significant HBsAg/IC's as early as
6/81 co-existent with low level anti-HBs, which
increased until HBV DNA clearance and then declined
rapidly (Figure 2B, middle panel). Similarly, this
20   patient evidenced significant anti-HBe antibody
production as early as 6/81, which persisted
throughout the observation period, and low level
HBeAg/IC's (Figure 2C, bottom panel).

        A second CAH patient (E.I.) who possessed similar
25   conventional serology as patient N.Y. (Figure 3A,
upper panel), demonstrated HBeAg/IC's and anti-HBe
antibodies 5 years prior to seroconverting to the
HBeAg-negative, anti-HBe-positive phase as determined
by conventional assays (Figure 3B, bottom panel).
30   Note the decline of HBeAg/IC's at the time of HBV DNA
clearance and the reciprocal rise in free anti-HBe.
Resolution of HBV infection always appears to be
preceded by the appearance of antibodies to HBeAg
measured directly as free anti-HBe or by the presence
35   of HBeAg/IC's (manuscript in preparation).

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In summary, the antigen-specific IC assays of
this invention represent valuable additions to the
serological analysis of chronic HBV infection. This
method of IC detection is clearly not limited to the
5   HBV system. It is anticipated that the solid-phase IC
method may be applied to the detection of IC's
containing other pathogen-derived or self antigens
provided that suitable anti-peptide antibodies with
the requisite characteristics can be developed. The
10   necessary characteristics simply are that the
anti-peptide antibody must bind soluble native antigen
efficiently, and must not compete for binding with
antibody raised against the native protein.
Antibodies with these characteristics can be produced
15   by methods other than immunization with synthetic
peptides. For example, immunizations with denatured
antigen may also result in the production of
non-competing, native-reactive antibodies. However in
this circumstance the production of monoclonal
20   antibodies would be required because of the polyclonal
nature of the response. The preselected specificity
of an anti-peptide response may preclude the necessity
for monoclonal antibody production.

25   The foregoing specification, including the
specific embodiments and examples, is intended to be
illustrative of the present invention and is not to be
taken as limiting. Numerous other variations and
modifications can be effected without departing from
30   the true spirit and scope of the present invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT:
        (A) NAME: The Scripps Research Institute
        (B) STREET: 10666 North Torrey Pines Road
        (C) CITY: La Jolla
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        (G) TELEPHONE: 619-554-2937
        (H) TELEFAX: 619-554-6312

    (ii) TITLE OF INVENTION: METHODS FOR THE DETECTION OF
            ANTIGEN-SPECIFIC IMMUNE COMPLEXES

    (iii) NUMBER OF SEQUENCES: 2

    (iv) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

    (v) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER: PCT/US93/
        (B) FILING DATE: 14-OCT-1993

    (vi) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: US 07/961,119
        (B) FILING DATE: 14-OCT-1992

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 13 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (v) FRAGMENT TYPE: internal

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

    Thr Thr Pro Ala Gln Gly Asn Ser Met Phe Pro Ser Cys

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     1                    5                    10
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(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 16 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (v) FRAGMENT TYPE: internal

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp Leu Val Val Ser Cys
1                   5                   10                  15
```

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What is Claimed is:

1.   A method for detecting the presence of an immunocomplex having antibody molecules complexed with a preselected antigen in a sample comprising the steps of:

    a)   admixing the sample with a detecting antibody molecule to form an immunoreaction admixture, said detecting antibody molecule capable of immunoreaction with said immunocomplex at a preselected site of said immunocomplex;

    b)   maintaining said immunoreaction admixture under immunoreaction conditions sufficient for said detecting antibody molecule to immunoreact with said immunocomplex and form a detecting antibody-immunocomplex reaction product;

    c)   detecting the presence of said detecting antibody-immunocomplex reaction product, and thereby said immunocomplex.

2.   The method of claim 1 wherein said detecting antibody molecule is in the solid phase.

3.   The method of claim 1 wherein said sample is a body fluid sample selected from the group consisting of blood and serum.

4.   The method of claim 1 wherein said preselected antigen is selected from the group consisting of bacterial, viral, parasitic, fungal, tumor and self antigen.

5.   The method of claim 4 wherein said viral antigen is selected from the group of viruses consisting of hepatitis B virus (HBV), human immunodeficiency virus, influenza A virus, Epstein-Barr virus, togavirus and rubella virus.

6.   The method of claim 5 wherein said HBV antigen is HBsAg or HBeAg.

7.   The method of claim 1 wherein said detecting

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antibody immunoreacts with the hepatitis B virus (HBV)
antigen HBsAg and immunoreacts with a polypeptide
having an amino acid residue sequence shown in SEQ ID
NO 1.

5   8.   The method of claim 1 wherein said detecting
antibody immunoreacts with the hepatitis B virus (HBV)
antigen HBeAg and immunoreacts with a polypeptide
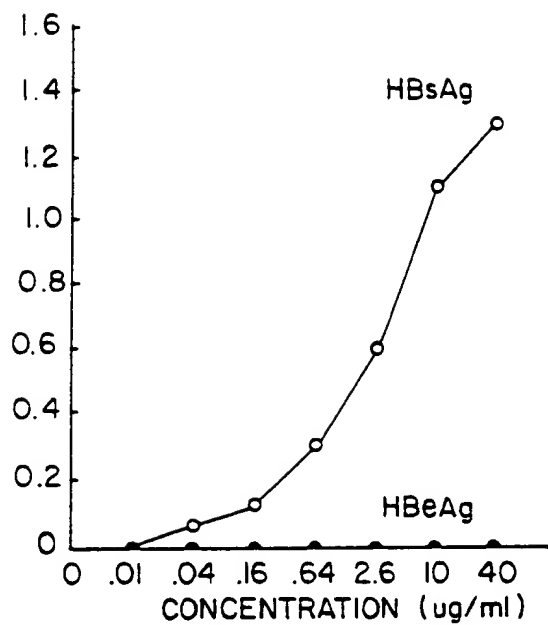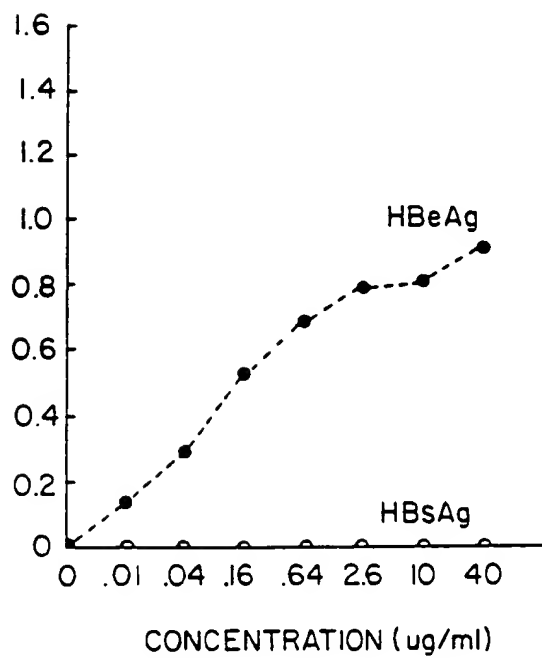having an amino acid residue sequence shown in SEQ ID
NO 2.

10

FIG. 1A

FIG. 1B

FIG. 2A

HBV DNA	+	+	+	+	+		±	-	-	-
HBe Ag	+	+	+	+	+		-	-	-	-
a-HBe (Abbott)	-	-	-	-	-		-	+	±	+
HBsAg	+	+	+	+	+		+	+	+	+
a-HBs (Abbott)	-	-	-	-	-		-	-	-	-
ALT	19	22	3	54	2		65	3	8	4

FIG. 2B
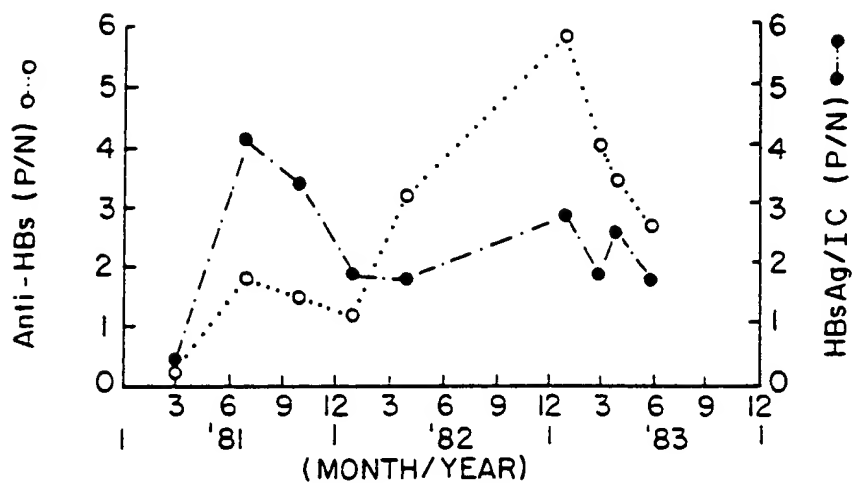
FIG. 2C
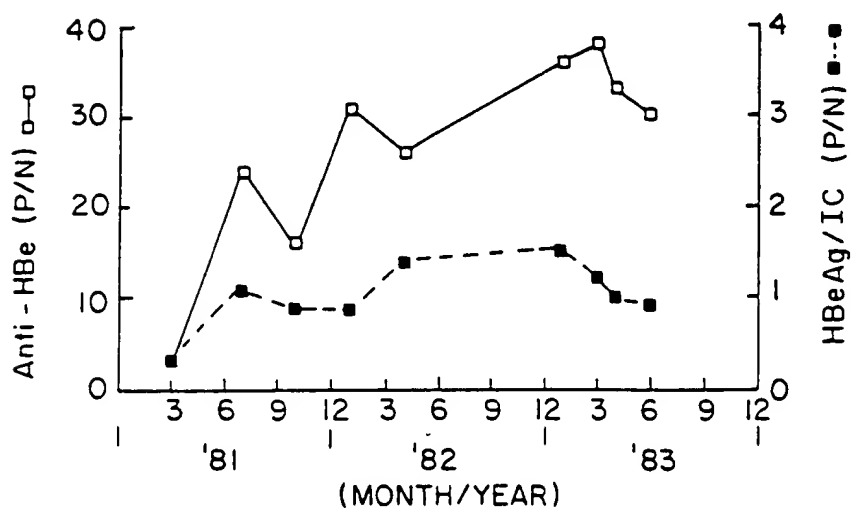
FIG. 3A

HBV DNA	++		+++	++	++	+	−	−
HBeAg	+		+	+	+	±	−	−
a-HBe (Abbott)	−		−	−	−	−	−	+
HBsAg	+		+	+	+	+	+	+
a-HBs (Abbott)	−		−	−	−	−	−	−
ALT	14		4	9	8	42	27	11

FIG. 3B
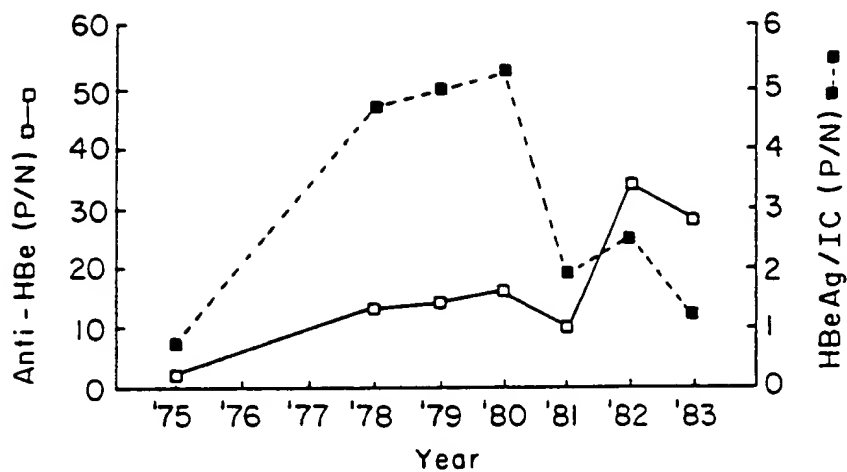
INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5, 7.1, 7.22, 7.23, 7.31, 7.92; 436/506, 507, 518, 820, 528; 530/387.9, 388.3, 389.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, CA; A-GeneSeq 12, PIR-38, Swiss-Prot 26

search terms: immune complex?, hepatitis, hbv, ebv, hiv, rubella, parasit?, influenza, epitope/

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	CLINICAL AND EXPERIMENTAL IMMUNOLOGY, Volume 72, issued 1988, S. Rath et al, "IgG Subclass Composition of Antibodies to HBsAg in Circulating Immune Complexes From Patients with Hepatitis B Virus Infections", pages 164-167, see entire document, especially page 165, Col 1.	1-6 7,8

☒ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
06 January 1994	26 JAN 1994
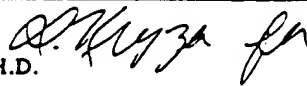
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer JAMES L. GRUN, PH.D.
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, Volume 79, issued September 1982, D.A. Shafritz et al, "Monoclonal Radioimmunoassays for Hepatitis B Surface Antigen: Demonstration of Hepatitis B Virus DNA or Related Sequences in Serum and Viral Epitopes in Immune Complexes", pages 5675-5679, see entire document, especially page 5677, Col 1.	7
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, Volume 78, Number 2, issued February 1981, J.R. Wands et al, "Immunodiagnosis of Hepatitis B with High-Affinity IgM Monoclonal Antibodies", pages 1214-1218, see pages 1214-1215, especially Table 1.	7
Y	JOURNAL OF VIROLOGY, Volume 63, Number 2, issued February 1989, J. Salfeld et al, "Antigenic Determinants and Functional Domains in Core Antigen and e Antigen from Hepatitis B Virus", pages 798-808, see entire document, especially page 804, Col 1.	8
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, Volume 79, issued July 1982, P.K. Bhatnagar et al, "Immune Response to Synthetic Peptide Analogues of Hepatitis B Surface Antigen Specific for the a Determinant", pages 4400-4404, see entire document, especially page 4402, Table 2.	7
Y	NATURE, Volume 281, issued 25 October 1979, F. Galibert et al, "Nucleotide Sequence of the Hepatitis B Virus Genome (Subtype ayw) Cloned in E. coli", pages 646-650, see especially Figure 3.	8
Y	EP, A, 0,044,710 (LERNER ET AL) 27 January 1982, see entire document.	7,8
X	US, A, 4,544,640 (SOMA ET AL) 01 October 1985, see entire document, especially: Col 1-4; Col 8, lines 38-68; Claims 2, 4, 22.	1-4

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

A61K 35/16; C07K 15/28; G01N 33/53, 33/543, 33/564, 33/569, 33/574, 33/576

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/5, 7.1, 7.22, 7.23, 7.31; 436/506, 507, 518; 530/387.9, 388.3, 389.4